BIOCHEMISTRY OF WATERLOGGED SOILS. PART III[1].

DECOMPOSITION OF CARBOHYDRATES WITH SPECIAL REFERENCE TO FORMATION OF ORGANIC ACIDS.

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(With Eight Text-figures.)

In the previous communications (25, 26) the more prominent effects of waterlogging in absence of foreign materials were described. The present paper deals with a study of the changes that attend addition of nitrates and carbohydrates.

Effect of addition of nitrates.

Warington's experiment (29) on percolation of nitrates through waterlogged soil was repeated with a view to determining whether any denitrification occurred under such conditions. Nitrates, after collection, were estimated by the Devarda alloy method (24). It was observed that in absence of plant residues nitrates could be completely recovered by repeated percolation, though the high concentration made the soil sticky and difficult to work with.

Table I.

Nitrates as p.p.m. of nitrogen.

Exp. No.	Soil	Days ...	0	1	3	5	7
I	Rothamsted	...	18·4	17·4	20·1	17·2	20·0
	Indian ...	...	18·4	16·9	15·4	13·6	18·8
II	Rothamsted	...	36·7	35·2	32·5	31·4	35·1
	Indian ...	...	36·7	32·7	26·5	29·1	30·4
III	Rothamsted	...	55·2	53·9	50·8	52·7	56·9
	Indian ...	...	55·2	49·5	47·2	52·6	47·7
IV	Rothamsted	...	73·5	72·1	74·8	72·3	71·9
	Indian ...	...	73·5	68·4	72·4	66·9	65·5
V	Rothamsted	...	92·1	83·8	78·7	81·2	76·1
	Indian ...	...	92·1	79·5	79·8	81·5	79·0

Average diminution ($\bar{x}$) ÷ standard error = $t^{(6)}$: t for 0–1 day = 3·5, which is significant.

In order to determine whether concentration of the nitrate had any effect on its subsequent transformation in the waterlogged soil, potassium nitrate was added in aqueous solution to 100 gm. lots of the same soils as those previously studied, to correspond to different concentrations

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and the mixtures were incubated, the Rothamsted soil at 20° C. and the Indian soil at 35° C., after being waterlogged. Samples were analysed at two-day intervals (Table I).

Though nitrates suffered a slight loss at the end of the 1st day, yet a simultaneous determination of total nitrogen indicated no change, thereby proving that no denitrification had occurred.

Effect of addition of fermentable organic matter.

A study of the transformation of organic matter is of fundamental importance and will throw light on the mode of decomposition of (*a*) straw, leaves, etc. which occur in the soil, (*b*) green manures and different other forms of organic fertilisers that are generally allowed to rot in the puddled soil prior to transplanting of paddy, and (*c*) various mineral transformations resulting in increase of plant food that occurs under such conditions. But it is exceedingly difficult to carry out because of (*a*) the complex composition of soil, (*b*) enormous number and variety of soil microflora, each member of which carries out its own characteristic function, and (*c*) want of adequate technique to deal either with the mixed flora or the different products of their metabolism as obtained under waterlogged conditions.

Among the different methods for studying biological activity in the soil the one introduced by Remy (21) and extended by Lohnis (14) is defective because the reactions are allowed to proceed in artificial media which do not represent natural soil conditions. The technique of Withers and Fraps (30) also introduces abnormal conditions because of the use of sterilised soil. The Lipman-Brown (13) and Russell-Hutchinson (23) methods are useful in the study of the release of available plant food, the former from added materials and the latter from unavailable forms present in the soil. In the present study an attempt was made to combine the Russell and Lipman methods with some modifications. Where adequate chemical techniques were lacking new ones were introduced. To avoid complexity due to indeterminate compositions of the added materials the following series of trials were carried out mainly with carbohydrates of well-defined composition.

Effect of added glucose on nitrates.

Glucose was added in solution to correspond to 600, 1200, 1800, 2400 and 3000 parts respectively as carbon per million parts of the soils which were waterlogged as usual (25). Nitrates were determined every 2 days (Table II).

It was observed that even at the end of 24 hours the soils developed a characteristic odour, and were frothy with carbon dioxide, and acid to phenolphthalein even after boiling. At later stages the changes were more pronounced.

Table II.

Nitrates in the Rothamsted and the Indian soils, at the beginning, were 16·1 and 43·8 p.p.m. respectively.

Nitrates as p.p.m. of nitrogen.

Glucose as p.p.m. of carbon	Days ...	Rothamsted soil 1	3	5	7	Indian soil 1	3	5	7
None		13·4	12·1	10·8	11·7	39·6	41·1	38·6	40·7
600		6·6	2·1	3·5	1·8	16·4	4·7	3·4	1·8
1200		3·0	...	...	...	6·8	2·1	1·6	...
1800		...	...	...	...	3·2	...	...	...

... nil; 2400 and 3000 p.p.m. of carbon—nil throughout.

In order to observe whether addition of glucose caused corresponding losses in total nitrogen a similar series of determinations were made with 30 gm. lots of soils. The results, however, showed that the changes were at no time significant. It should, therefore, be inferred that the rapid disappearance of nitrates was not due to denitrification but to transformation to other forms of nitrogen.

DISSOLVED OXYGEN.

In order to determine whether addition of sugar introduced any change in the oxygen-contents, trials were carried out under the same conditions as in the previous experiments. Since, owing to the constant interchange of oxygen between the soil-sediment and the surface water (25), an increase or decrease in the one would be correspondingly reflected in the other, it was considered sufficient to determine only the amounts of oxygen present in surface water. Rideal and Stewart's modification (22)

Table III.

Oxygen at the beginning in both soils = 7·3 p.p.m.

Dissolved oxygen as p.p.m.

Glucose as p.p.m. of carbon	Days ...	Rothamsted soil 1	3	5	7	Indian soil 1	3	5	7
None		7·9	8·7	7·0	8·4	5·8	6·1	5·2	5·6
600		1·6	5·3	5·8	8·0	1·1	2·4	5·3	6·3
1200		1·3	2·8	3·7	4·9	1·4	1·2	0·2	2·4
1800		1·0	1·1	2·1	2·6	0·7	1·0	1·3	1·7
2400		0·5	0·4	1·3	1·7	0·3	0·4	0·8	1·0
3000		0·2	nil	0·4	1·0	nil	0·2	0·6	0·6

of Winkler's method was adopted with correction for dissolved reducing matter. The results (Table III) showed that the oxygen-contents first fell considerably, but soon rose owing to diffusion of oxygen from air. The recovery was rapid when the sugar added was small, but slow when large.

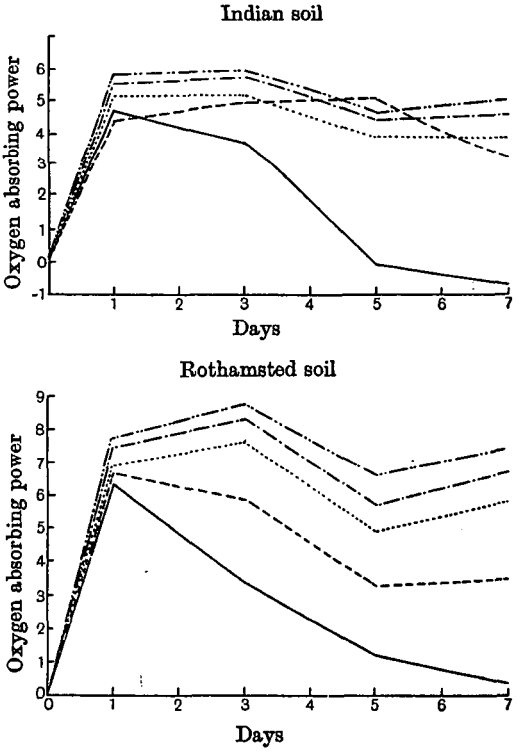


Fig. 1. Oxygen absorbing power.

———— Soil + 600 p.p.m. of carbon. – – – – – – Soil + 1200 p.p.m. of carbon.
..... Soil + 1800 ,, —·—·— Soil + 2400 ,,
—··—··— Soil + 3000 p.p.m. of carbon.

The addition of sugar led evidently to some internal reaction which caused rapid depletion of oxygen. At later stages oxygen of the air diffused in and restored normal conditions. Since the soils remained exposed to air, oxygen in accordance with the gas laws would have diffused into them only in proportion to their shortage from the normal. "Oxygen absorbing powers" of the soils thus estimated (Fig. 1) measured also, indirectly, the intensity of biological action that was mostly, if not entirely, responsible for the depletion of oxygen.

Carbon dioxide present, dissolved in the surface water, was determined

by absorption in baryta and back-titration against standard acid. The data (Fig. 2), though not absolute measures of the gas produced, were estimates of the concentrations developed at different stages. They are positively correlated to the corresponding figures for oxygen absorbing power and indicate that oxygen was largely utilised for the biological oxidation of the carbohydrate to carbon dioxide.

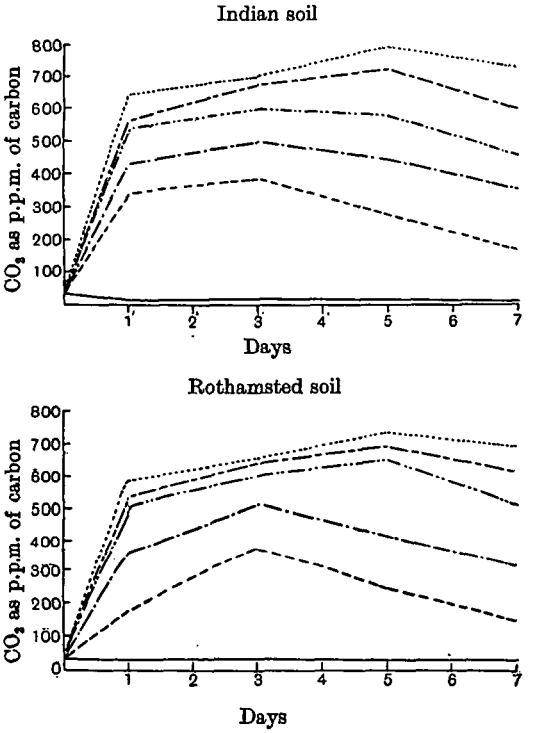


Fig. 2. Dissolved carbon dioxide.

——————— Soil alone.
– – – – – – – – „ + 600 p.p.m. of fermentable carbon.
—·—·—·— „ +1200 „ „
—··—··— „ +1800 „ „
— – — – — – „ +2400 „ „
..... „ +3000 „ „

The reaction of aqueous extracts of soils to which sugar had been added, as determined by Gillespie's method (8), indicated a slight initial change (*p*H 7·5 to 6·5) to acidity. It returned to normal on standing.

Tests were carried out with clarified extracts of soils for the presence of organic acids. Lactic acid was detected by its reduction of permanganate and chromic acid with evolution of acetaldehyde, and by

iodoform, and Fletcher and Hopkins'(7) tests. Acetic and butyric acids were also identified in the liquid by a variety of tests(5).

Bacterial numbers.

Bacterial numbers were determined by plating on Thornton's medium(28). It was observed (Fig. 3) that in addition to the great

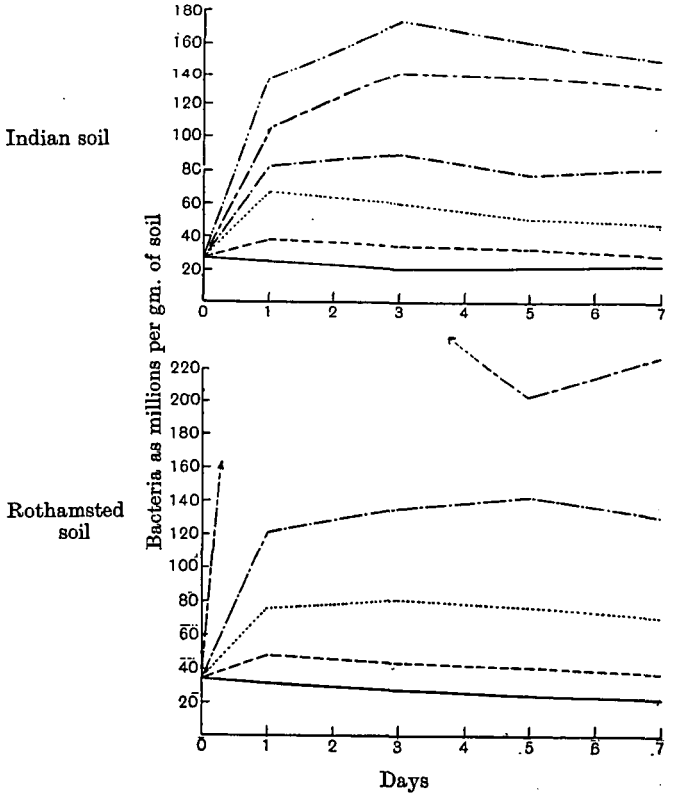


Fig. 3. Bacterial numbers.

——————— Soil alone.
– – – – – – – – „ +600 p.p.m. of carbon.
. . . . . „ +1200 „
—·—·—·— Soil +1800 p.p.m. of carbon.
— – — – — – „ +2400 „
—··—··— „ +3000 „

increase in bacteria, higher concentrations (2400 and 3000 p.p.m.) of the sugar brought out enormous numbers of fungi which rendered counting difficult. The decrease in numbers noticed after the 3rd day might have been due to both shortage of the sugar and to the distinctly visible increase in protozoa.

Correlation between bacterial and oxygen absorption numbers, though positive, was not very close, thereby suggesting that all the bacteria did not depend on oxygen. Many of those responding to the higher concentrations of the sugar might have been either strict or facultative anaerobes. The closer agreement observed between bacterial numbers and CO_2-production lent further support to this view.

ASSIMILATION OF NITRATES BY SOIL ORGANISMS IN PRESENCE OF GLUCOSE.

Soils treated with 600 p.p.m. of the sugar were plated out on Giltay's Agar. After incubation for 7 days at 35° C. the colonies coming out on the plates were examined. The organisms common to all of them, but morphologically different from each other, were inoculated into sterile suspensions of both the soils containing identical amounts of sugar and nearly the same quantities of nitrate by the necessary additions and developed as seed cultures. After they had developed for 24 hours they were transferred into larger quantities of similar soil suspensions. The specimens were incubated for 3 days at 35° C. and then analysed for their nitrate contents (Table IV).

Table IV.

Nitrates as p.p.m. of nitrogen.

	Rothamsted soil		Indian soil		
Designation of the organism used	Present after 3 days	Amount used up	Present after 3 days	Amount used up	Differences between the amounts used up from two soils
A (R)	28·4	42·9	36·6	36·0	+6·9
B	34·1	37·2	41·8	30·8	+6·4
C	37·3	34·0	45·0	27·6	+6·4
D	67·6	3·7	69·2	3·4	[+0·3]
E	23·3	48·0	32·7	39·9	+8·1
A (I)	40·8	30·5	51·3	21·3	+9·2
B	22·7	48·6	30·1	42·5	+6·1
C	69·4	1·9	68·7	3·9	[−2·0]
D	20·9	50·4	30·3	42·3	+8·1
E	27·2	44·1	35·6	37·0	+7·1

Nitrate originally present in R (Rothamsted soil) = 71·3 p.p.m. and I (Indian soil) = 72·6 p.p.m.

Most of the samples had then turned cloudy with bacterial growth. Some of them had developed characteristic odours. No frothing or gas-production was visible. Except in one case the extracts were all acid to phenolphthalein even after boiling.

The results show that (*a*) nitrate assimilation is common to most soil

organisms, (*b*) where the response of an organism to nitrate is pronounced, the effect of the soil substrate is about the same as instanced by the nearly constant differences (7·3 ± 1·1) observed between the amounts of nitrate assimilated in presence of the two soils, and (*c*) the study of single species of organisms, however prominent, will not be representative: further investigation should, therefore, be carried out with the mixed microflora of the soils.

Relation between concentration of nitrate and the rate of its decomposition in presence of glucose.

100 gm. suspensions of the soils containing 600 p.p.m. of glucose but different proportions of nitrate were incubated, the Rothamsted soil at 20° C. and the Indian soil at 35° C., for 24 hours. They were then analysed for their nitrate-contents and the quantities decomposed by the microflora in each case determined (Table V).

Table V.

Nitrates as p.p.m. of nitrogen.

Rothamsted soil		Indian soil	
Originally present	Decomposed	Originally present	Decomposed
16·1	9·3	43·8	26·7
34·5	19·4	62·2	33·6
52·8	32·1	80·5	41·1
71·3	49·3	99·0	50·8
89·6	65·5	117·3	51·4
108·2	65·1	—	—

The rate of decomposition of nitrate increases with concentration up to a maximum. It is higher for the Rothamsted soil than for the Indian in spite of the lower temperature of incubation and bears evidence to the greater biological activity in the former.

Distribution of carbon and nitrogen in waterlogged soils.

Simultaneous changes in different forms of nitrogen and carbon were studied, using suspensions of soils containing glucose (600 p.p.m.) and nitrate (89·6 p.p.m. in the Rothamsted and 99·0 in the Indian specimens respectively). The soils were waterlogged and incubated under conditions similar to those in the previous series for 48 hours and samples taken for analyses every 12 hours. The determinations were not carried out over a longer period because it was observed that most of the changes took place within 2 days.

Residual sugar at different stages was estimated by a modified Fehling titration method (27): total CO_2 by absorption in baryta, with boiling to drive off that present in the surface water: nitrite by the Davisson method (4): nitrate by the Devarda alloy method (24): ammonia

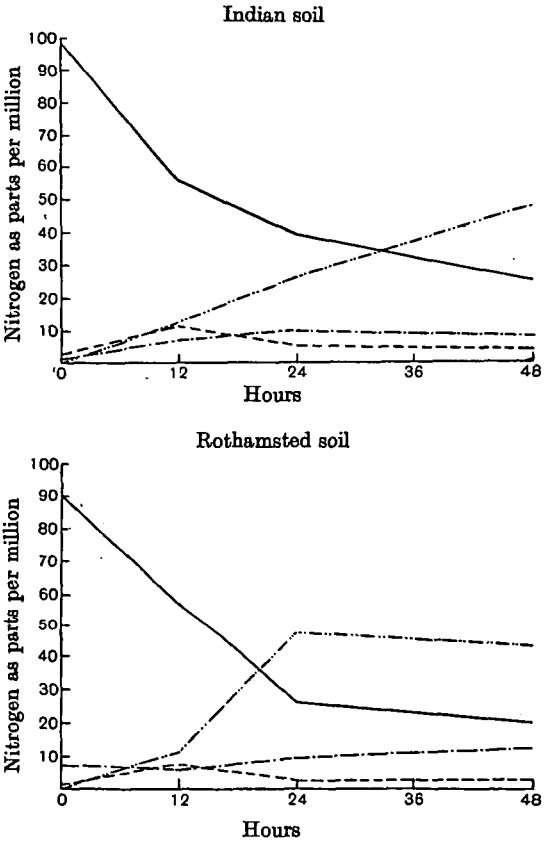


Fig. 4. Distribution of nitrogen.

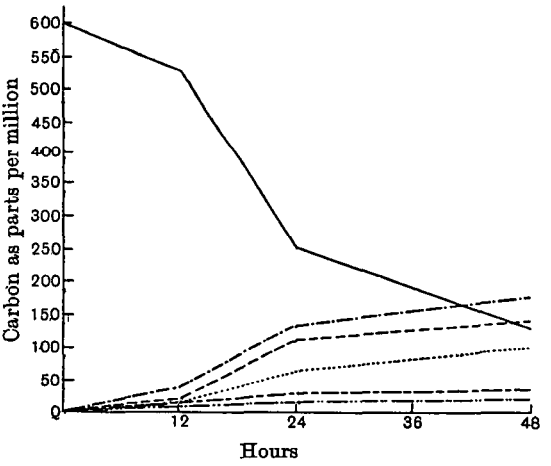
——————— Nitrogen as nitrates. - - - - - - - - - Nitrogen as nitrites.
—·—·—·— ,, as ammonia. —··—··— ,, assimilated by microflora.

by the McLean and Robinson method (19): total nitrogen by the Gunning-Hubbard method (1): lactic acid by the Buchner and Meisenheimer method (3) after concentrating the neutralised aqueous extract to a small volume: and acetic and butyric acids by steam-distilling at constant volume (27).

CHANGES IN DIFFERENT FORMS OF NITROGEN (Fig. 4).

As was expected nitrates disappeared rapidly: nitrites increased at the same time, suggesting that they were formed mainly from them.

Indian soil



Rothamsted soil

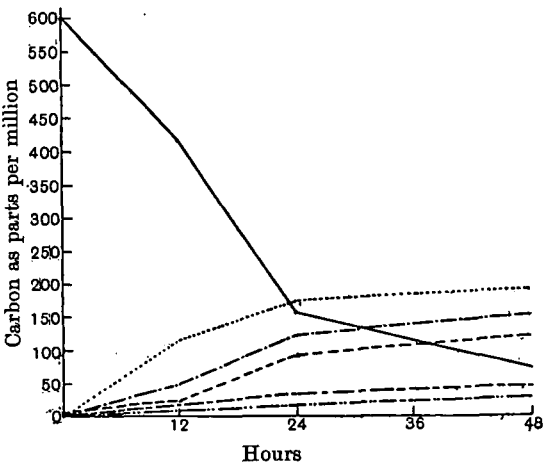


Fig. 5. Distribution of carbon.

———— Carbohydrate added. – – – – – – – – CO_2 produced.
—·—·—·— Lactic acid formed. — — — — — — Acetic acid formed.
—··—··— Butyric acid formed. ····· Carbon taken up by micro-organisms.

Formation of nitrite accords with the observations of Nagaoka (20), Kelly (11) and others. The subsequent decrease in nitrites may have been

due to (*a*) reduction to ammonia, (*b*) direct assimilation by micro-organisms, and (*c*) denitrification. Since total nitrogen remained unaltered throughout the period under observation it should be inferred either that there was no loss by denitrification, or that it was too small to be appreciable. There was significant increase in ammonia in both soils which may have been due to (*a*) the action of the deaminising enzyme present in the soils (26), and (*b*) reduction of nitrates and nitrites by ammonifiers (17).

Nitrogen taken up by micro-organisms or otherwise converted into more complex forms was taken to be approximately the difference between the sums of nitrate, nitrite and ammonia present in the beginning and at the end. The figures showed that the major part of the added nitrogen was thus converted. Since the diminution in the quantities of the nitrates and nitrites present occurred simultaneously with the increased uptake by the micro-organisms and since no other changes were noticeable, it appeared that most of the two soluble forms were thus converted. Since nitrogen of cells of micro-organisms is essentially protein it seems probable that most of the transformed nitrogen was converted into that form.

CHANGES IN DIFFERENT FORMS OF CARBON (Fig. 5).

In both soils the decomposition of the added sugar was extremely rapid. About one-fourth to one-fifth of the total added carbon passed into CO_2. Production of lactic acid accounting for between 25 and 30 per cent. of sugar in both soils was an important change, and may have been due largely to lactic acid bacteria and, to a limited extent, to certain putrefactive and saprophytic organisms like *B. coli* and *B. lactis aerogenes* present in soil. Only small quantities of acetic and butyric acids were formed during the period under investigation. Their molecular proportion was approximately as two of acetic to one of butyric. Quantities of the added carbon converted into complex forms by cells of micro-organisms or otherwise were taken as represented by differences between initial and subsequent amounts present in simpler forms. The figures show that such transformations are greater in Rothamsted than Indian soil. No definite relationship could be found between carbon and nitrogen assimilations.

GENERAL DISTRIBUTION OF THE DECOMPOSED CARBON.

Dividing carbon changes under four heads—(*a*) total converted, (*b*) passing into gas, (*c*) taken up by micro-organisms, (*d*) passing into organic acids—it is evident that the last change is not only unique to the

waterlogged soil, but also the most prominent, involving nearly half the added carbon. The production of acids, particularly lactic, though beginning comparatively slowly, soon proceeded at a rapid rate. Even at the end of 48 hours there was no indication of the reaction weakening.

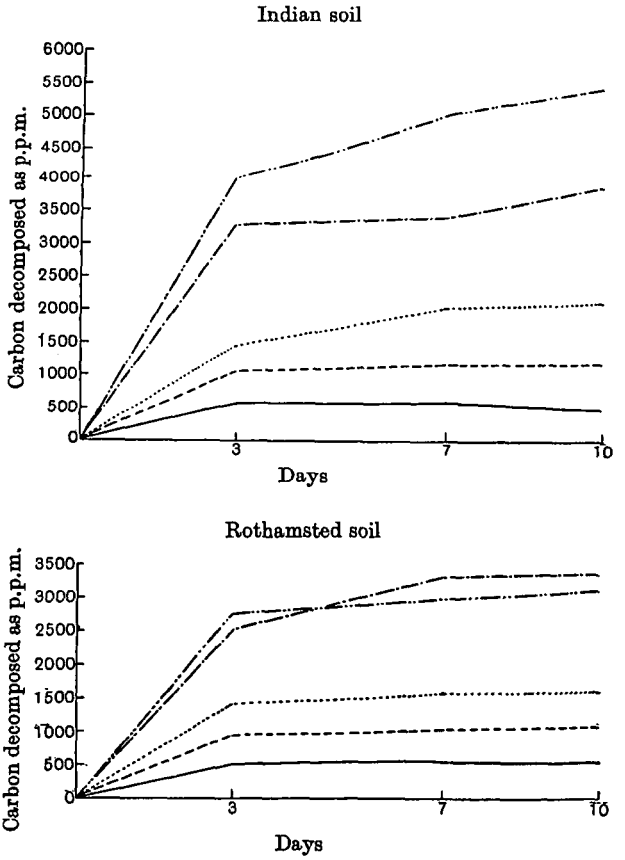


Fig. 6. Effect of concentration on decomposition of fermentable carbon.

——————— 600 p.p.m. added carbon. —·—·—·— 6000 p.p.m. added carbon.
– – – – – – 1200 „ —··—··— 12,000 „
····· 2400 p.p.m. added carbon.

Since, however, it is known that many of the common bacteria and fungi can not only assimilate organic acids and their salts but also decompose them into simpler compounds(16), the presence of large quantities of organic acids would naturally mean that further biological activity would follow later on. Acid-production would thus be only an intermediate stage in a long series of reactions.

DECOMPOSITION OF ADDED CARBOHYDRATE AT DIFFERENT CONCENTRATIONS.

To specimens of Rothamsted and Indian soils glucose was added from aqueous solution to correspond to 600, 1200, 2400, 6000, and 12,000 p.p.m. of carbon, and after waterlogging incubated for 10 days at 20° C.

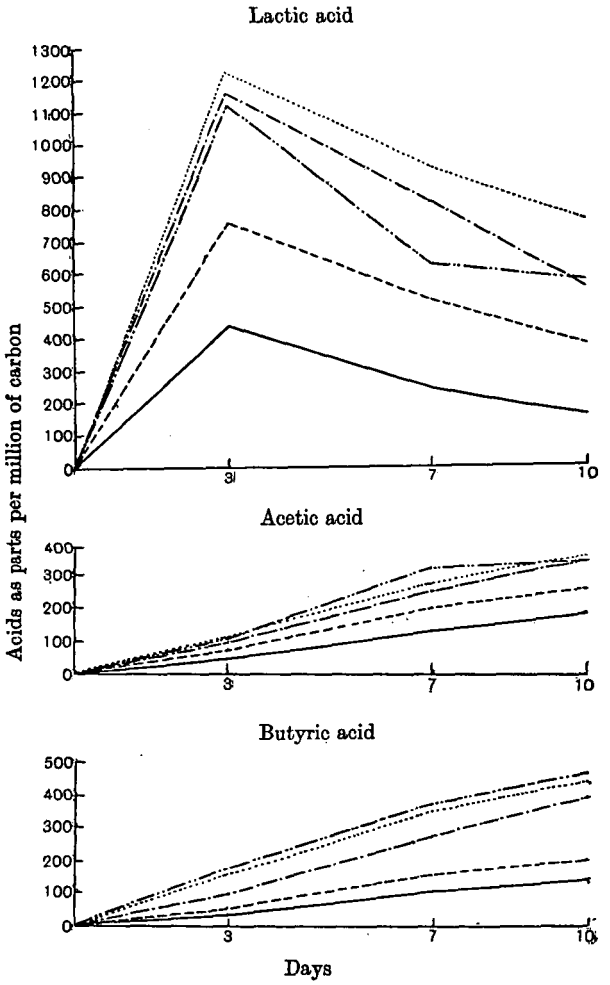


Fig. 7. Formation of lactic, acetic and butyric acids—Rothamsted soil.

——————— 2400 p.p.m. fermentable carbon.
– – – – – – 4800 ,,
—·—·—·— 7200 ,,
····· 9600 ,,
—··—··— 12,000 ,,

and 35° C. respectively. Samples were analysed every 3 days for their sugar-contents (27).

The results (Fig. 6) showed that decomposition of sugar of all concentrations proceeded vigorously up to the end of the 3rd day, after which there was general slackening. This was probably due to shortage of sugar at lower concentrations and accumulation of acids and other products of biological metabolism at higher ones. Total quantities decomposed were, proportionately, much less at higher concentrations than at lower ones.

Production of organic acids at different concentrations of added carbohydrate.

Soils were treated in the same manner as in the previous trial. Lactic acid was estimated by Long's method (15) and acetic and butyric acids by steam-distillation at constant volume (27). Similar types of results were obtained for both soils. Those for Rothamsted soil are plotted in Fig. 7.

At all concentrations of sugar lactic acid was the first product of metabolism and accounted for 30–40 per cent. of the sugar decomposed. After the 3rd day, however, lactic acid tended to diminish, being, evidently, converted into other forms.

Acetic and butyric acids were formed slowly but continuously throughout the period under observation. There was no relation between their quantities and the corresponding amounts of sugar decomposed. Molecular proportions of the two acids were approximately as two of acetic to one of butyric. There was high positive correlation between decrease in lactic and corresponding increase in the other two acids, suggesting that decomposition of the former led, at least partly, to formation of the latter.

Table VI.

Amounts of acids formed as p.p.m. of carbon.

Depth of water	Time in days	Lactic	Acetic	Butyric
Soil just covered	3	912	192	72
	7	516	384	216
	10	264	456	264
2″	3	1164	84	144
	7	432	312	324
	10	336	408	432
4″	3	1272	60	168
	7	684	288	384
	10	432	432	456

RELATION BETWEEN DEPTH OF WATER AND PRODUCTION OF ORGANIC ACIDS.

Specimens of Rothamsted soil containing 6000 p.p.m. of glucose were suspended under different depths of water and the acids formed at the end of 3, 7 and 10 days estimated (Table VI).

Greater depths led to larger production of lactic and butyric acids, suggesting that their formation did not depend on free supply of air. Diminished production of acetic acid under similar conditions suggested the reverse.

EFFECT OF ADDITION OF CALCIUM CARBONATE ON PRODUCTION OF ACIDS.

To 30 gm. portions of the soils containing 6000 p.p.m. of glucose, 2 gm. of calcium carbonate were added before waterlogging. The acids formed were estimated at the end of 3, 7 and 10 days (Table VII).

Table VII.

Amounts of acids as p.p.m. of carbon.

Treatment	Time in days	Rothamsted soil			Indian soil		
		Lactic	Acetic	Butyric	Lactic	Acetic	Butyric
Without $CaCO_3$	3	1164	100	95	1224	118	139
	7	816	252	276	494	384	516
	10	552	348	396	256	432	624
With $CaCO_3$	3	1536	118	121	1788	152	166
	7	745	334	451	991	387	494
	10	374	392	506	716	451	577

Addition of calcium carbonate led to distinct increase in lactic acid: subsequent decomposition of the acid was also rapid, particularly in Rothamsted soil.

The effect of carbonate on the production of the other acids was not pronounced.

PRODUCTION OF ORGANIC ACIDS DURING AN EXTENDED PERIOD OF OBSERVATION.

Specimens of the Rothamsted soil containing 6000 p.p.m. of glucose were analysed for their acid-contents at intervals during a period of 30 days.

It was observed (Fig. 8) that lactic acid decomposed actively and fairly uniformly. Acetic and butyric acids, after increasing till about the 15th day, decomposed appreciably at later stages. The results support

the theory, already suggested, that the acids would, on standing, be attacked by micro-organisms and converted into other forms.

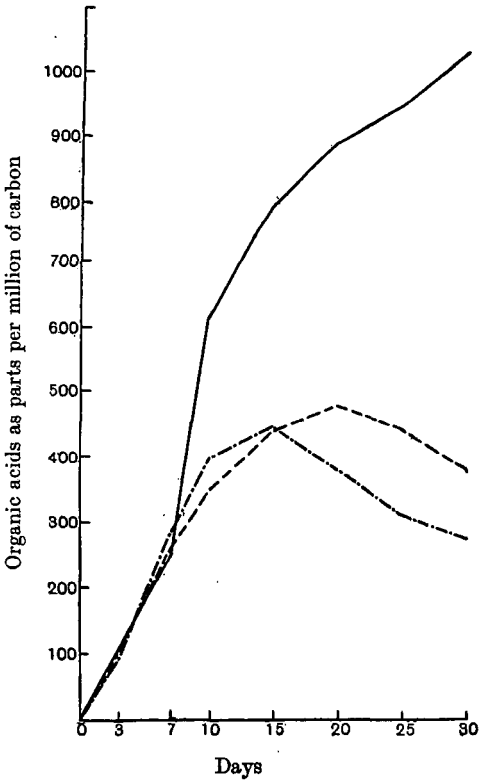


Fig. 8. Changes in organic acids over a long period.

——————— Lactic acid decomposed. – – – – – – Acetic acid produced.
—•—•—•— Butyric acid produced.

Production of lactic acid under aerobic and anaerobic conditions.

20 gm. lots of Rothamsted soil were waterlogged with 4000 p.p.m. of glucose. In one set the mixture was allowed to stand in shallow, glass-covered trays. In the other, after being made up with air-free water, it was placed in narrow-necked flasks connected with U-tubes containing alkaline pyrogallol. Both sets were maintained at the ordinary temperature (20–25° C.). Lactic acid present on successive days was estimated (Table VIII).

Table VIII.

Lactic acid formed as p.p.m. of carbon.

Time in days	Aerobic: Control soil and water alone	Aerobic: With the sugar	Anaerobic: Control soil and water alone	Anaerobic: With the sugar
1	27	378	45	567
2	54	907	63	855
3	45	1134	54	1071

Production of lactic acid occurred under both aerobic and anaerobic conditions and to about the same extent.

FORMATION OF LACTIC ACID IN ABSENCE OF LIVING ORGANISMS.

20 gm. lots of Rothamsted soil were weighed out into dishes with glass covers and allowed to remain soaked in toluene at the ordinary temperature for 48 hours, after which 50 c.c. of distilled water and 20 c.c. portions of 1 per cent. solutions of glucose, sucrose and lactose respectively were added to them. The mixtures were allowed to stand for 3 days. Lactic acid in this and the subsequent series was estimated by the author's modification (27) of the chromic acid method (Table IX).

Table IX.

Lactic acid formed as p.p.m. of carbon.

Time in days	Control soil alone	Glucose	Sucrose	Lactose
1	27	108	189	72
2	54	207	243	189
3	54	234	297	216

It is probable that part of the lactic acid formed during waterlogging was enzymic in origin. The major part, however, was evidently formed by the activity of living organisms.

MECHANISM OF PRODUCTION OF LACTIC ACID.

Since the acid was formed under both aerobic and anaerobic conditions, its production did not involve the utilisation of atmospheric oxygen. Nor is it probable that any biological reduction of the sugar was involved since the acid is a more highly oxygenated product than the sugar. The chemical mechanism was probably either hydrolysis or direct splitting of the carbohydrate. Though Hoppe-Seyler (10) and later Kiliani (12) showed that lactic acid can be formed by the hydrolysis of certain carbohydrates, yet the conditions of their experiments bear no analogy to the production of the acid in the waterlogged soil. Since it

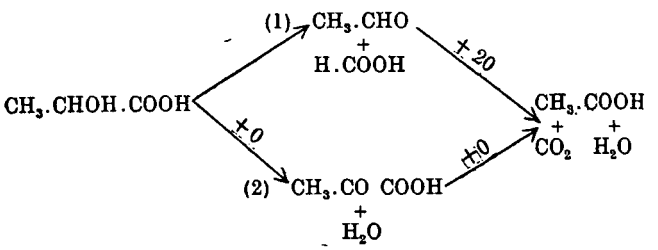
was also observed on trial that the acid was not formed by acid-hydrolysis of the sugars even at 100° C. it may be inferred that hydrolysis was not the mechanism of its production. Biological production of lactic acid was therefore probably one of fission of the carbohydrate molecule which did not bear any relation to the reactions brought about by common chemical reagents. Formation of the acid from glucose would then be represented in outline by $C_6H_{12}O_6 \longrightarrow 2C_3H_6O_3$. The primary change involved in its production would therefore appear to be intramolecular rearrangement of the sugar.

Production of acetic and butyric acids from lactic acid.

In order to test whether any of the fatty acids were formed directly from lactic acid, quantities of the latter corresponding to 1200 p.p.m. of carbon were added to the Rothamsted soil and waterlogged under aerobic and anaerobic conditions. The former was effected by gentle bubbling of air through the suspension and the latter by using air-free water and connecting the flasks to U-tubes containing alkaline pyrogallol. The fatty acids were identified and estimated at intervals of 24 hours (Table X).

The extent of decomposition of lactic acid was about the same under aerobic and anaerobic conditions. Under anaerobic conditions the greater part of it passed into fatty acids, while only less than half did so under the aerobic.

The quantities of acetic acid produced under aerobic conditions were uniformly greater than those produced anaerobically. In the former it was formed almost to the entire exclusion of butyric acid and appeared to have been mainly due to oxidation of lactic acid which might have been through the stages of (1) acetaldehyde or (2) pyruvic acid. Both types of reactions might also have taken place simultaneously.



Qualitative analyses made from time to time showed only the presence of pyruvic acid, as indicated by the nitroprusside, β-naphthol and phenylhydrazine reactions. Positive reactions for the aldehyde

could at no time be obtained. As shown by Maze and Ruot(18) it is probable that certain fungi brought about the oxidation of lactic into pyruvic acid.

Table X.

Acids as p.p.m. of carbon.

Time in days	Aerobic			Anaerobic		
	Lactic	Acetic	Butyric	Lactic	Acetic	Butyric
1	984	96	24	996	48	132
2	816	264	48	744	168	276
3	552	216	36	624	192	336

Attempts at reproducing the chemical oxidation of lactic acid at concentrations similar to those obtained in the waterlogged soil to pyruvic acid by the Aristoff method(2) resulted only in the formation of acetaldehyde as the intermediate product. The biological formation of pyruvic acid which proceeds under nearly neutral conditions (*p*H 6·5) has evidently a different mechanism.

Production of the fatty acids seems to proceed along different lines under anaerobic conditions. Neither pyruvic acid nor aldehyde could be detected at any time.

The molecular proportion under fully aerobic conditions was as 12 of acetic to 1 of butyric and, under anaerobic, approximately as 1 : 1. Since in the waterlogged soil it was 2 : 1 it should be inferred that the conditions therein were intermediate between the above two.

ORGANISMS RESPONSIBLE FOR THE DIRECT ASSIMILATION OF LACTIC ACID.

To isolate the organisms directly assimilating lactic acid the soils were plated out on a medium containing lactic acid (0·2 per cent.), soil extract (sp. gr. 1·002), agar (2·0 per cent.) and a few drops of brom-cresol-purple indicator. After incubation for 10 days at the ordinary temperature the organisms turning the medium purple (*i.e.* destroying the acid and thereby rendering the medium neutral) were examined.

The plates from the Rothamsted soil contained a large number of colonies of a micrococcus which produced the purple within a week. There were also rapidly growing colonies of a *Penicillium* which did not produce the colour till the 10th day. Plates from the Indian soil also contained the *Penicillium*, but the coccoid forms were not to be found. On inoculating the two organisms into lactic acid (45 milligrams) diluted with soil extract (100 c.c., sp. gr. 1·002) it was observed that the acid was rapidly assimilated (Table XI).

Table XI.

Lactic acid assimilated in mg.

Organism	3 days	7 days
Micrococcus	9·7	14·1
Penicillium	12·0	28·7

No acetic or butyric acid was formed at the same time. Qualitative examination showed that the mucilaginous development of the coccus was a carbohydrate resembling glycogen in iodine reaction.

PRODUCTION OF ORGANIC ACIDS FROM DIFFERENT CARBOHYDRATES.

Specimens of the Rothamsted soil were treated with (1) glycerol, (2) xylose, (3) arabinose, (4) laevulose, (5) mannitol, (6) sucrose, (7) maltose, (8) lactose, (9) starch, (10) maltodextrin and (11) cellulose (cotton-wool), each in quantities corresponding to 6000 p.p.m. of carbon, waterlogged at the laboratory temperature (20–25° C.). The acids formed at different stages were estimated (Table XII).

Table XII.

Organic acids as p.p.m. of carbon.

No.	Time in days	Lactic	Acetic	Butyric
1	3	67	35	82
	7	112	58	61
2	3	314	46	76
	7	88	118	62
3	3	1126	200	204
	7	272	498	292
4	3	978	272	331
	7	421	326	484
5	3	1187	18	278
	7	321	86	543
	10	119	401	446
6	3	679	126	320
	7	214	333	513
7	3	1688	540	291
	7	796	762	161
8	3	892	306	112
	7	264	416	268
9	3	382	102	101
	7	204	128	62
10	3	436	115	251
	7	196	85	234

Minute quantities of the acids were also formed from cellulose. Formation of the acids was generally accompanied by production of carbon dioxide. The quantities of acetic and butyric acids did not bear

any relation to each other or to decomposed lactic acid. Though the same acids were formed in every case their modes of formation appeared to vary.

SUMMARY.

(1) In absence of decomposing organic matter addition of nitrate led to no loss of nitrogen.

(2) On addition of small quantities of fermentable matter such as glucose there was (*a*) rapid depletion of nitrates and oxygen, but no denitrification, and (*b*) increase in acidity, carbon dioxide and bacteria. The greater part of the soluble nitrogen was assimilated by micro-organisms or otherwise converted and the greater part of the added carbohydrate was transformed into lactic, acetic and butyric acids.

(3) The organic acids were formed from a variety of carbohydrates. Lactic acid was the first to be observed and appeared to be formed mainly by direct splitting of the sugar. It decomposed readily, forming acetic and butyric acids. Some acetic acid was formed by direct oxidation of lactic acid, with pyruvic acid as the intermediate product. All the acids were, on standing, converted into other forms by micro-organisms.

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